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## PAPER

# The effect of pegylated recombinant human leptin (PEG-OB) on weight loss and inflammatory status in obese subjects

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**OBJECTIVE:** To investigate whether weekly subcutaneous administration of 60 mg of long-acting pegylated human leptin (PEG-OB) for 8 weeks was able to influence weight loss, metabolic profile and inflammatory status of obese subjects on a mildly hypoenergetic diet (deficit: 3.2 MJ/day).

**DESIGN:** A prospective, randomized, double-blind and placebo-controlled single-center trial.

**SUBJECTS:** Twenty-eight healthy, obese subjects (16 women, 12 men; age 22–65 y; body mass index 27.7–38.7 kg/m<sup>2</sup>).

**MEASUREMENTS:** Bodyweight, metabolic profile (including lipids), C-reactive protein (CRP) and soluble TNF  $\alpha$ -receptor (sTNF-R) 55 and 75 levels.

**RESULTS:** At the end of the study no significant differences in the delta or percentage weight loss between the placebo ( $n=14$ ) and PEG-OB ( $n=14$ ) groups was observed. Also the changes in metabolic profile, CRP, sTNF-R55 and R75 concentrations between the two groups after 8 weeks of treatment did not differ.

**CONCLUSION:** Weekly injection of 60 mg PEG-OB did not lead to additional weight loss after 8 weeks of treatment. Furthermore, PEG-OB administration did not affect the changes in metabolic profile and the inflammatory status of obese subjects.

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**Keywords:** PEG-OB; leptin; obesity; C-reactive protein; tumor necrosis factor; chronic systemic inflammation

## Introduction

Leptin, the protein product of the *ob* gene, plays an important role in the regulation of food intake and energy expenditure in animal models via hypothalamic mechanisms.<sup>1–3</sup> Treatment of normal and diet-induced obese mice with recombinant leptin or long-acting pegylated recombinant leptin (PEG-OB) results in decreased food intake and weight loss, although the latter group requires a higher dose, indicating relative leptin resistance.<sup>4</sup> The first clinical trial conducted by Heymsfield and coworkers observed a dose–

response relationship with weight and fat loss in both lean and obese humans after exposure to recombinant human leptin supporting this concept.<sup>5</sup> These early results suggested that high therapeutical doses of human recombinant leptin might be able to reduce body weight in obese subjects. In contrast, we failed to show an effect of PEG-OB administration on body weight or energy expenditure in obese subjects.<sup>6</sup> However, it should be noted that the dose used in the latter study (20 mg per week) only led to 60% of the maximal serum leptin concentrations observed in the highest dose cohorts of the study of Heymsfield. Hence, the administration of a higher dose of PEG-OB might be able to affect body weight in obese subjects.

The discovery that the leptin receptor is widely expressed in various non-neuronal tissues including lymph nodes, macrophages and hemopoietic cells suggests that leptin

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also possesses other extrahypothalamic effects.<sup>7–9</sup> Several lines of evidence indicate that leptin has immunoregulatory actions. The leptin receptor is structurally related to the class I cytokine receptor family and has been shown to have signaling capabilities of interleukin 6-type cytokine receptors.<sup>10</sup> Rodents with defects at different sites in the leptin-dependent signaling pathway exhibit increased sensitivity to endotoxin-induced lethality, deficits in macrophage phagocytosis, impaired T-cell function and lymphoid atrophy. Exogenous leptin was able to correct the majority of these immune dysfunctions.<sup>11–14</sup> Moreover, leptin deficient human infants were reported to be more susceptible to infections.<sup>15</sup> *In vitro* experiments showed that leptin activated human peripheral monocytes by inducing proliferation and proinflammatory cytokine production in a dose dependent fashion. These results prompted investigators to suggest that leptin might have an additional physiological role as a proinflammatory cytokine.<sup>16</sup> Leptin levels are generally elevated in the obese population.<sup>17</sup> Furthermore, obesity appears to be associated with a state of low-grade systemic inflammation.<sup>18</sup> Together, it seems conceivable that increased leptin levels present in human obesity might contribute to this state of low-grade systemic inflammation.

The purpose of this study was to explore whether weekly administration of a high dose (60 mg per week) of long-acting pegylated human leptin (PEG-OB) was able to induce additional weight loss and metabolic changes in obese subjects on a mildly hypoenergetic diet. Furthermore, we investigated if PEG-OB was able to change the inflammatory status of these subjects by measuring concentrations of the acute-phase C-reactive protein (CRP) and soluble TNF  $\alpha$ -receptor (sTNF-R) 55 and 75 levels.

## Methods

### Subjects and study design

Twenty-eight healthy obese subjects (16 women, 12 men) were recruited by local advertising and studied after provision of written informed consent. The study was approved by the Medical Ethical Committee of the Maastricht University. Obese (body mass index (BMI)  $\geq 27.0$  kg/m<sup>2</sup>) subjects of 18–65 y old were eligible for inclusion. Female subjects had to be sterile or post-menopausal (1 y). Subjects with obesity-related diseases requiring pharmacological treatment (eg diabetes, hypertension, dyslipidemia) were excluded. Other exclusion criteria were: weight loss more than 3 kg in the previous 3 months, presence of any significant illness, including laboratory or electrocardiogram abnormalities, history or presence of drug abuse or alcoholism, smoking more than five cigarettes or equivalent per day. Also, known allergy, history of atopy or hypersensitivity to pegylated proteins and use of any drug that might have influenced body weight led to exclusion. This single-center trial had a prospective, randomized, double-blind and placebo-controlled group design. After a lead-in diet period lasting 4 weeks, only those subjects who lost 1.75 kg or more from

their initial body weight were allowed to continue to the treatment phase. Eligible subjects were subsequently stratified and matched into pairs according to gender, age, initial body weight, initial BMI and the amount of body weight lost during the lead-in diet period to achieve balanced treatment groups.

Randomization numbers for subjects were generated and incorporated into the double-blind labeling by an independent third party. Treatment consisted of 60 mg PEG-OB (6 ml, 10 mg/ml; produced and provided by Hoffmann-La Roche Inc., Nutley, NJ, USA) or matching placebo (6 ml) administered subcutaneously (s.c.) once a week in the para-umbilical region for a total of 8 weeks. In addition, all subjects were prescribed a mildly hypoenergetic diet during the lead-in and 8 week treatment period designed to reduce daily energy intake by 3200 kJ/day (800 kcal/day). The energy content of the diet was calculated from the patients' estimated basal metabolic rate multiplied by 1.6 to estimate the total daily energy expenditure.<sup>19</sup> From energy expenditure, 3200 kJ/day (800 kcal/day) was subtracted to obtain a mildly hypoenergetic diet. The dietary prescription was discussed every week with a dietician. Body weight was measured weekly on a calibrated digital scale accurate to 0.1 kg and height was measured to the nearest 0.01 m. The BMI was calculated as body weight (kg) divided by height (m) squared. Safety of PEG-OB was monitored weekly by documentation of adverse events and the recording of vital signs. Routine clinical hematology and biochemical tests and urine analysis were conducted throughout the study by the certified central laboratory of the University Hospital Maastricht, The Netherlands.

### Collection and analysis of blood samples

Blood samples were collected after an overnight fast on week-4 (start of the study), day 1 (start treatment period) and on week 8 (end of the study) and immediately cooled on ice (plasma) or allowed to clot at room temperature (serum). Plasma and serum were extracted by centrifugation (twice at 4°C), frozen in liquid nitrogen and stored at –80°C until further analysis. Plasma substrates were determined enzymatically in duplicate using the hexokinase method (Roche, Basel, Switzerland) for glucose, the Wako NEFA C kit (Wako Chemicals, Neuss, Germany) for FFA, the glycerolkinase method (Boehringer Mannheim GmbH, Mannheim, Germany) for free glycerol, the lipase method (Sigma Diagnostics, St Louis, MO, USA) for triglycerides, and the CHOD-PAP method (Boehringer Mannheim GmbH, Mannheim, Germany) for cholesterol. To avoid interassay variability, all specimens for a given substance were run in a single assay.

Insulin serum levels were measured by ELISA (Mercodia insulin ELISA; Mercodia AB, Uppsala, Sweden). Insulin resistance was estimated using the HOMA-R method.<sup>20</sup> Total leptin concentrations (endogenous leptin plus PEG-OB) were measured according to the method described previously.<sup>6</sup>

Plasma concentrations of both soluble TNF $\alpha$ -receptors and CRP levels were measured using specific sandwich ELISAs. sTNF-R55 and sTNF-R75 were detected as described elsewhere.<sup>21</sup> Plasma CRP levels were measured using an ELISA made up with a polyclonal antibody to human CRP that was used both as control and detector of CRP. The intra- and interassay coefficients of variations were both below the 10%. All measurements were performed in duplicate.

### PEG-OB

Recombinant methionyl human leptin has a reported average terminal half-life of approximately 4 h in humans, which requires daily administration to obtain sustained blood levels.<sup>5</sup> Modification of proteins through covalent linkage of polyethylene glycol polymers to the protein has resulted in reduced immunogenicity and increased serum half-life for a number of proteins. Recombinant native human leptin, expressed and purified from *Escherichia coli*, was chemically conjugated to a species of branched polyethylene glycols (PEG) with an average molecular weight of 42 kDa in a 1:1 ratio. The result was a globular PEG-native human leptin polymer (PEG-OB) with increased molecular size. PEG-OB at a concentration of 10 mg/ml was placed in sterile glass vials containing 1.3 ml. Preclinical studies with PEG-OB indicate an extended half-life (> 48 h) and efficacy for reduction of food intake and body weight in animals.<sup>22</sup> Our previous study in obese male subjects clearly showed sustained elevated blood levels following weekly s.c. dosing of PEG-OB in humans. Mean peak serum PEG-OB concentrations were achieved 72 h after dosing followed by a return to the elevated pre-dose levels after 1 week.<sup>6</sup>

### Statistical analysis

Changes from baseline after 8 weeks treatment were compared between the PEG-OB treated and placebo group using factorial ANOVA. *Post hoc*, for each comparison separately, ANOVA with repeated measures was used. Additional statistical tests were used when appropriate. All statistical tests were two-sided and significance was defined as  $P < 0.05$ . All data are presented as mean  $\pm$  s.e.m. unless otherwise indicated.

### Results

The baseline demographic characteristics of the subjects are shown in Table 1. The characteristics of the 14 subjects randomized to each treatment group were similar. All 28 subjects that were randomized into the two treatment groups completed the trial. No subjects were excluded at the end of the lead-in diet period for dietary non-compliance. The most common adverse events related to treatment were injection site ecchymosis, pruritis and pain (Table 2). These occurred with similar frequency in both the placebo and the PEG-OB groups. No difference was detected between

**Table 1** Baseline demographics

Characteristic	Placebo (n = 14)	PEG-OB (n = 14)
Sex		
Male	6	6
Female	8	8
Age (y)	52.7 $\pm$ 3.2 (22.0–65.0)	49.6 $\pm$ 3.3 (28.0–64.0)
Weight (kg)	95.4 $\pm$ 4.4 (68.4–135.5)	96.1 $\pm$ 4.1 (73.4–126.7)
Height (m)	1.68 $\pm$ 0.03 (1.49–1.87)	1.71 $\pm$ 0.03 (1.55–1.92)
BMI (kg/m <sup>2</sup> )	33.4 $\pm$ 0.8 (27.7–38.7)	32.7 $\pm$ 0.6 (29.0–36.0)
Body weight loss during lead-in period (kg)	5.1 $\pm$ 0.4 (3.1–8.0)	5.5 $\pm$ 0.5 (2.9–9.1)

Baseline characteristics of the 28 subjects at the screening.

BMI, body mass index.

No statistical significant differences were found between both groups.

Data are presented as mean  $\pm$  s.e.m. (range).

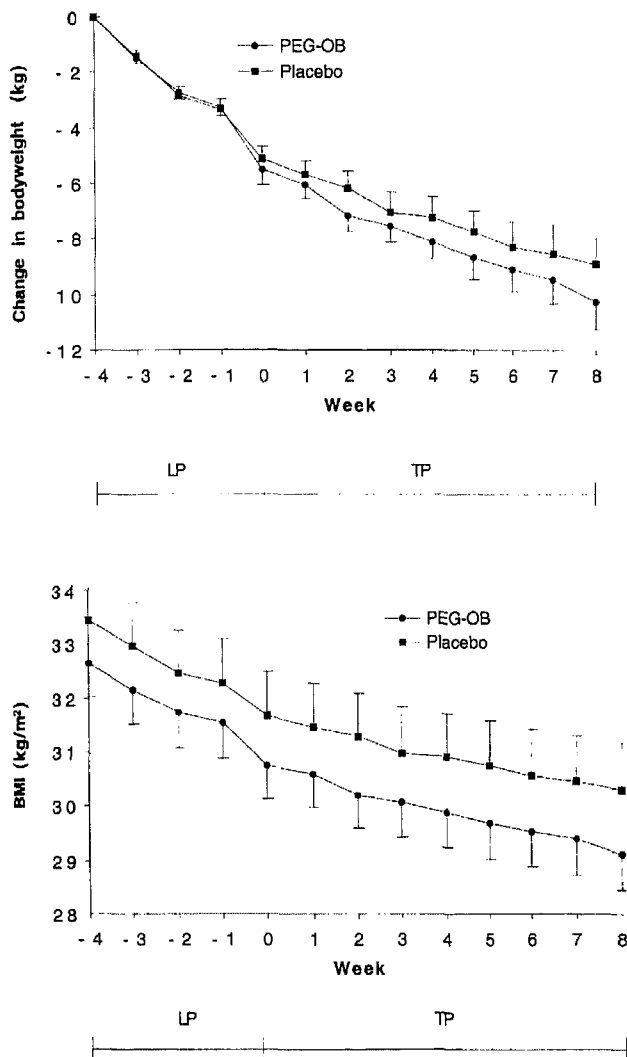
**Table 2** Most common adverse events related to treatment

Event	Frequency (no. of subjects reporting)	
	Placebo	PEG-OB
Hematoma at the injection site	6/14	5/14
Itching at the injection site	1/14	3/14
Pain at the injection site	1/14	2/14

the groups with regards to vital signs, standard chemistry or hematology assessments and urine analysis.

The effect of PEG-OB treatment or placebo treatment on body weight and BMI are illustrated in Figure 1. At the end of the lead-in period the mean body weight loss was  $-5.3 \pm 0.45$  kg in all 28 subjects studied (Table 1). After completion of the treatment period both the placebo ( $n=14$ ) and PEG-OB ( $n=14$ ) groups had lost a similar amount of weight (week 8 body weight: placebo  $86.5 \pm 4.3$  kg; delta  $-3.8$  kg; PEG-OB  $85.8 \pm 4.8$ ; delta  $-4.8$  kg;  $P=0.32$ ). There was no significant difference in the delta or percentage change in weight and BMI between the PEG-OB and placebo groups.

The effect of 60 mg PEG-OB or placebo treatment on the metabolic profile (including lipids) is shown in Table 3. Plasma glucose and serum insulin concentrations decreased throughout the study. However, no significant differences were observed between the treatment groups. Insulin resistance (estimated by the HOMA-R method) also showed no significant differences between both groups before and after treatment. No significant differences in plasma FFA, glycerol, total cholesterol and triglyceride concentrations were observed at the end of the 8 week treatment period and there were no differences between the treatment groups. Following weekly s.c. dosing of PEG-OB, elevated serum levels of total leptin (endogenous plus PEG-OB), measured a week after the last dose, ranging from 800–3900 ng/ml were observed.



**Figure 1** Mean change in bodyweight (a) and BMI (b) from the start of the lead-in period (LP) of 4 weeks until the end of the 8 week treatment

The result of PEG-OB or placebo treatment on the concentrations of CRP, sTNF-R55 and 75 are given in Table 4. Weight reduction during the lead-in period significantly decreased the levels of sTNF-R55 from  $0.37 \pm 0.02$  ng/ml to  $0.36 \pm 0.02$  ng/ml ( $P=0.03$ ) in the whole group, but did not affect the concentrations of sTNF-R75 and CRP. No significant differences in CRP, sTNF-R55 and sTNF-R75 concentrations were observed after 8 weeks of treatment between the groups.

## Discussion

The results show that exposure to 60 mg PEG-OB weekly for 8 weeks did not influence weight loss in obese subjects on a mildly hypoenergetic diet despite the high serum levels of PEG-OB achieved at the end of this study. Thus, augmentation of serum leptin concentration using a long-acting PEG-OB failed to promote additional weight loss over caloric restriction. The outcome of this study is, however, consistent with the alternative view of the physiological role of leptin proposed by the group of Flier.<sup>23</sup> These investigators suggest that evolution would favor a leptin dose-response curve that functions briskly as a switch between the fed and fasted state, but would fail to limit further energy storage as levels rose with increased energy stores. The latter state could be described as 'leptin resistance'. In addition, they speculate that the shape of this biological dose-response curve may depend on the conditions in which a certain species evolved. This hypothesis provides a possible explanation for the ineffectiveness of high-dose PEG-OB in our obese individuals on a mildly hypoenergetic diet as well for the fairly moderate results of the Heymsfield trial.<sup>5</sup> According to this view, PEG-OB might cause additional weight loss when administered during severe energy restriction or total leptin deficiency. The observation that recombinant human met-leptin treatment of a young hyperphagic very obese girl with a mutated *ob* gene resulted in weight loss by sustained reductions in appetite is consistent with this view.<sup>24</sup> The fact that

**Table 3** Effect of 8 weeks of treatment with PEG-OB or placebo on metabolic profile

	Start of lead-in		Day 1		Day 56	
	Placebo (n=14)	PEG-OB (n=14)	Placebo (n=14)	PEG-OB (n=14)	Placebo (n=14)	PEG-OB (n=14)
Glucose (mmol/l)	$5.54 \pm 0.07$	$5.54 \pm 0.13$	$5.19 \pm 0.08^a$	$5.14 \pm 0.12^a$	$5.30 \pm 0.10$	$5.24 \pm 0.13$
Insulin (mU/ml)	$16.8 \pm 2.7$	$15.8 \pm 2.5$	$9.8 \pm 1.6^a$	$12.2 \pm 2.3^a$	$9.1 \pm 1.8$	$8.5 \pm 1.5^b$
HOMA-R	$4.17 \pm 0.67$	$4.03 \pm 0.69$	$2.24 \pm 0.36^a$	$2.90 \pm 0.57^a$	$2.16 \pm 0.44$	$2.04 \pm 0.38^b$
FFA ( $\mu$ mol/l)	$780 \pm 60$	$786 \pm 52$	$777 \pm 73$	$682 \pm 44$	$399 \pm 30^b$	$428 \pm 37^b$
Glycerol ( $\mu$ mol/l)	$159.7 \pm 16.1$	$149.4 \pm 14.2$	$138.5 \pm 12.0$	$140.0 \pm 14.8$	$96.2 \pm 10.8^b$	$116.3 \pm 18.5$
Triglycerides (mmol/l)	$1.20 \pm 0.12$	$1.72 \pm 0.35$	$1.01 \pm 0.14^a$	$1.75 \pm 0.42$	$0.88 \pm 0.11$	$1.25 \pm 0.15$
Total cholesterol (mmol/l)	$5.63 \pm 0.42$	$5.91 \pm 0.37$	$4.76 \pm 0.31^a$	$5.51 \pm 0.36^a$	$5.10 \pm 0.31^b$	$5.46 \pm 0.34$
Total leptin <sup>c</sup> (ng/ml)	$18.4 \pm 2.8$	$17.4 \pm 2.5$	$11.2 \pm 1.8^a$	$11.6 \pm 1.7^a$	$10.9 \pm 1.7$	$2542 \pm 245^b$

Data are mean  $\pm$  s.e.m. HOMA-R, estimated insulin resistance (see method section).

<sup>a</sup>Significant difference day 1 vs start,  $P < 0.05$ .

<sup>b</sup>Significant difference day 56 vs day 1,  $P < 0.05$ .

<sup>c</sup>Note that the assay did not distinguish between endogenous leptin and PEG-OB. There were no significant differences between treatments over time.

Table 4 Effect of 8 weeks of treatment with PEG-OB or placebo on the inflammatory status

	Start of lead-in		Day 1		Day 56	
	Placebo (n = 14)	PEG-OB (n = 14)	Placebo (n = 14)	PEG-OB (n = 14)	Placebo (n = 14)	PEG-OB (n = 14)
sTNF-R55 (ng/ml)	0.37 ± 0.03	0.38 ± 0.03	0.36 ± 0.03	0.36 ± 0.03	0.37 ± 0.03	0.36 ± 0.03
sTNF-R75 (ng/ml)	1.26 ± 0.09	1.26 ± 0.09	1.21 ± 0.10	1.30 ± 0.09	1.24 ± 0.08	1.37 ± 0.11
CRP (mg/l)	4.31 ± 1.34	5.97 ± 1.13	4.47 ± 2.44	5.88 ± 1.17	3.39 ± 1.33	5.28 ± 0.88

Data are mean ± s.e.m.

sTNF-R55, soluble TNF $\alpha$ -receptor 55; sTNF-R75, soluble TNF $\alpha$ -receptor 75; CRP, C-reactive protein.

<sup>a</sup>Significant difference day 1 vs start,  $P < 0.05$ .

<sup>b</sup>Significant difference day 56 vs day 1,  $P < 0.05$ .

There were no significant differences between treatments over time.

supraphysiological levels of PEG-OB cause weight loss in rodents<sup>22</sup> but not in humans suggests that the leptin dose-response curves of these species are different.

However, the possibility that the small number of subjects studied and the relative short duration of treatment might explain the lack of an effect of PEG-OB treatment on weight loss in this study cannot be excluded.

Also other biological effects of leptin in animals studies were not observed in this study.<sup>4</sup> We failed to demonstrate any treatment effects on the levels of glucose, insulin (including estimated insulin resistance) and triglycerides. These results are supported by data obtained from the limited number of human patients with leptin deficiency or non-functional leptin receptors studied up to now who also lack substantial impairments in glucose homeostasis and lipids (unlike, respectively, the *ob/ob* and *db/db* mice) suggesting that leptin is not directly involved in the regulation of these systems in man.<sup>15,25</sup> Our previous study suggested that PEG-OB treatment might have an additional effect on triglycerides in obese subjects consistent with similar changes repeatedly observed in animal studies.<sup>6</sup> In the present study with a higher dose of PEG-OB no added effect on triglycerides was observed.

Next, we studied whether administration of longacting pegylated human leptin affects the levels of soluble TNF $\alpha$ -receptors (sTNF-R55 and 75) and CRP. Soluble TNF $\alpha$  receptor levels have been validated as sensitive indicators of activation of the TNF $\alpha$  system.<sup>26</sup> CRP is an acute phase protein of the pentraxin family and a sensitive marker for systemic inflammation. Our data showed that administration of pegylated human leptin to obese subjects affected neither the levels of sTNF-R55 and 75 nor those of CRP, suggesting that leptin is not directly involved in the enhanced inflammatory status present in obesity. Thus, it seems plausible that the definite long-term elevations in systemic leptin and CRP found in obesity result from a common pathogenic mechanism present in this condition.

In summary, weekly s.c. administration of 60 mg PEG-OB to obese subjects on a mildly hypoenergetic diet did not affect weight loss, metabolic profile and inflammatory status after 8 weeks. In addition, PEG-OB treatment was generally well tolerated and safe in these subjects.

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